

Phylogenetic and taxonomic classification of the *Microtus (Terricola) grafi* based on samples from the Balkan Peninsula using ancient mtDNA

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Introduction:

Within the genus *Microtus* (Arvicolinae, Rodentia), the subgenus *Terricola* is recognised, which includes approximately 13 to 16 species [1–3]. The most widespread among them is the European pine vole [*M. (T.) subterraneus*] [2] (Fig. 1a, 1b), while others are restricted to smaller regions [1]. Due to the low morphological diversity within the genus *Microtus*, unambiguous systematic classification can be problematic [4, 5]. In 1992, a new morphological form was discovered at the Late Pleistocene Bacho Kiro site (Bulgaria). Based on the unique pattern of the occlusal surface of the first molar, intermediate between *M. (T.) multiplex* and *M. (T.) subterraneus*, a new species, *Microtus (Terricola) grafi*, was described [6] (Fig. 1c).

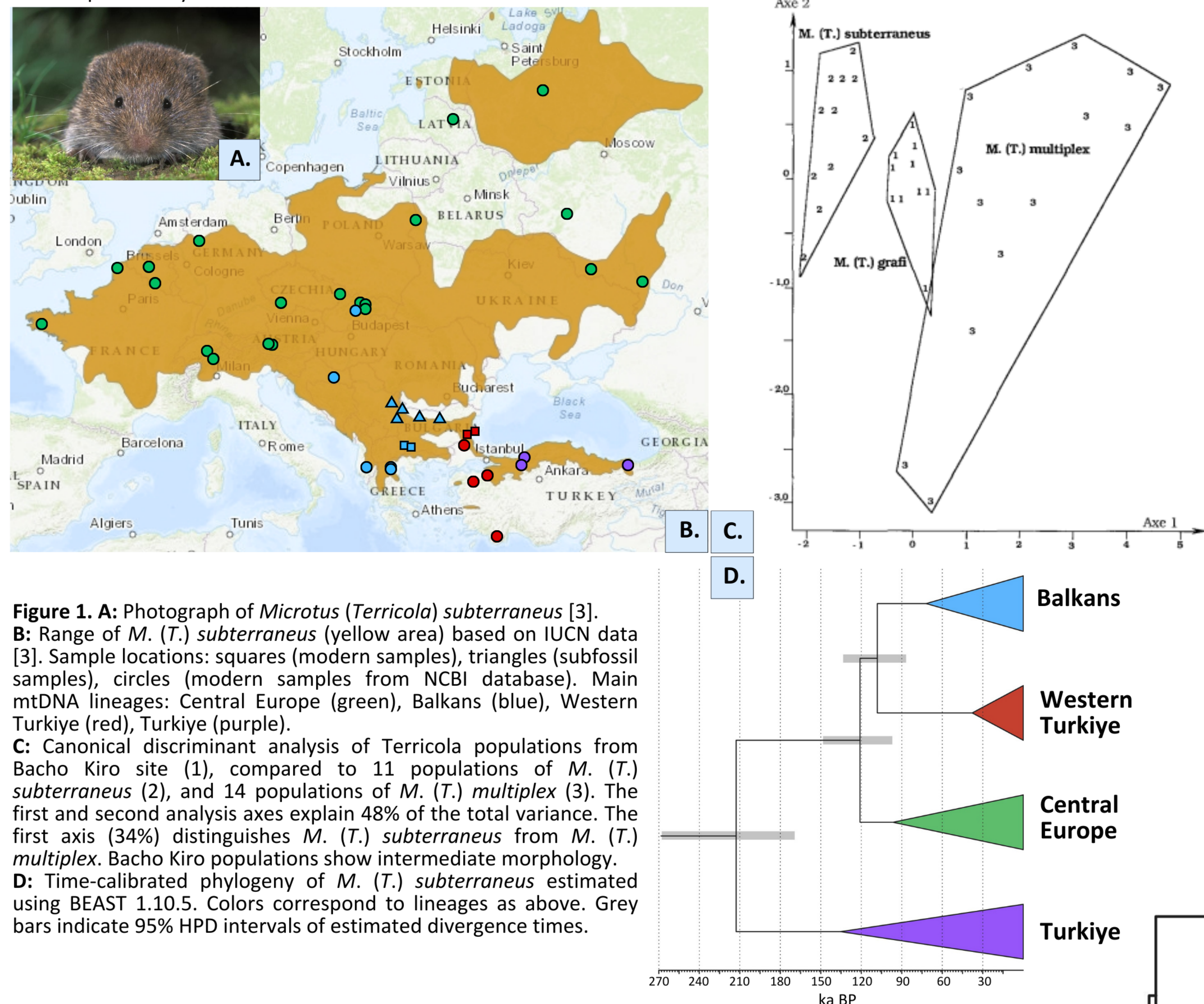
This study aimed to verify the taxonomic status of *Microtus (Terricola) grafi* and to determine its phylogenetic relationships with other species of the subgenus *Terricola* by analyzing cytochrome *b* (mtDNA) sequences from subfossil samples from Late Pleistocene sites and modern samples from the Balkans.

Results:

- The mtDNA genome sequences of 19 ancient and 8 modern samples were reconstructed.
- The samples previously classified as *M. (T.) grafi* (all from the Bacho Kiro site) fall within the genetic variation of *M. (T.) subterraneus*. Most ancient samples are clustered within the clade formed by modern specimens from the Balkan area (Fig. 2).
- Samples of the species *M. (T.) subterraneus* form four main clades: Central Europe, Balkan, Turkiye, and Western Turkiye.
- We estimated the divergence times of main clades: the Turkiye clade – approx. 214 ka BP (95% HPD: 270–140 ka BP), Western Turkiye and Central Europe with the Balkan Peninsula – approx. 122 ka BP (95% HPD: 100–150 ka), and Balkan and Central Europe – approx. 109 ka BP (95% HPD: 87–130 ka) (Fig. 1d).
- The estimated divergence times of the main clades coincide with MIS 7 and MIS 5 interglacials and are similar to estimates obtained for a range of rodents, including collared lemmings, narrow-headed voles, common voles and field vole [7–9].

Conclusions:

- We suggest that individuals previously classified as *M. (T.) grafi* should be considered part of the morphological variation within the species *M. (T.) subterraneus*.
- The simultaneous divergence of different species during MIS 7 and MIS 5 suggests large-scale environmental changes that affected European ecosystems.



Methodology:

DNA extraction followed the protocol of Rohland et al. (2018) for 25 subfossil samples identified as *Microtus (Terricola) grafi* (from Bacho Kiro site) or *Microtus Terricola* sp. and 13 modern samples identified as *Microtus Terricola* sp. Single-stranded DNA libraries were prepared from DNA extracts using the protocol of Gansauge et al. (2020). These libraries were then enriched in vole mtDNA, following the protocol of Horn (2012), using bait produced from modern specimens. Double-stranded libraries were prepared for modern samples. Due to low sequence quality, single-stranded libraries were also prepared for some modern samples using the same protocol. Sequencing was performed using the Illumina platform. The resulting sequences were mapped to mtDNA reference genomes of selected *Terricola* species [13, 14]. We reconstructed the phylogenetic tree using the Maximum Likelihood approach in IQ-TREE 2 (cyt) with ultrafast bootstrapping to assess node support [15] and the Bayesian method in MrBayes 3.2.7a [16]. Initial alignments included sequences from various *Terricola* species; we then added 53 published cytochrome *b* sequences of *M. (T.) subterraneus* (Fig. 2) from different European localities (Fig. 1b). To estimate divergence times and sample ages, we used BEAST 1.10.5 [17].

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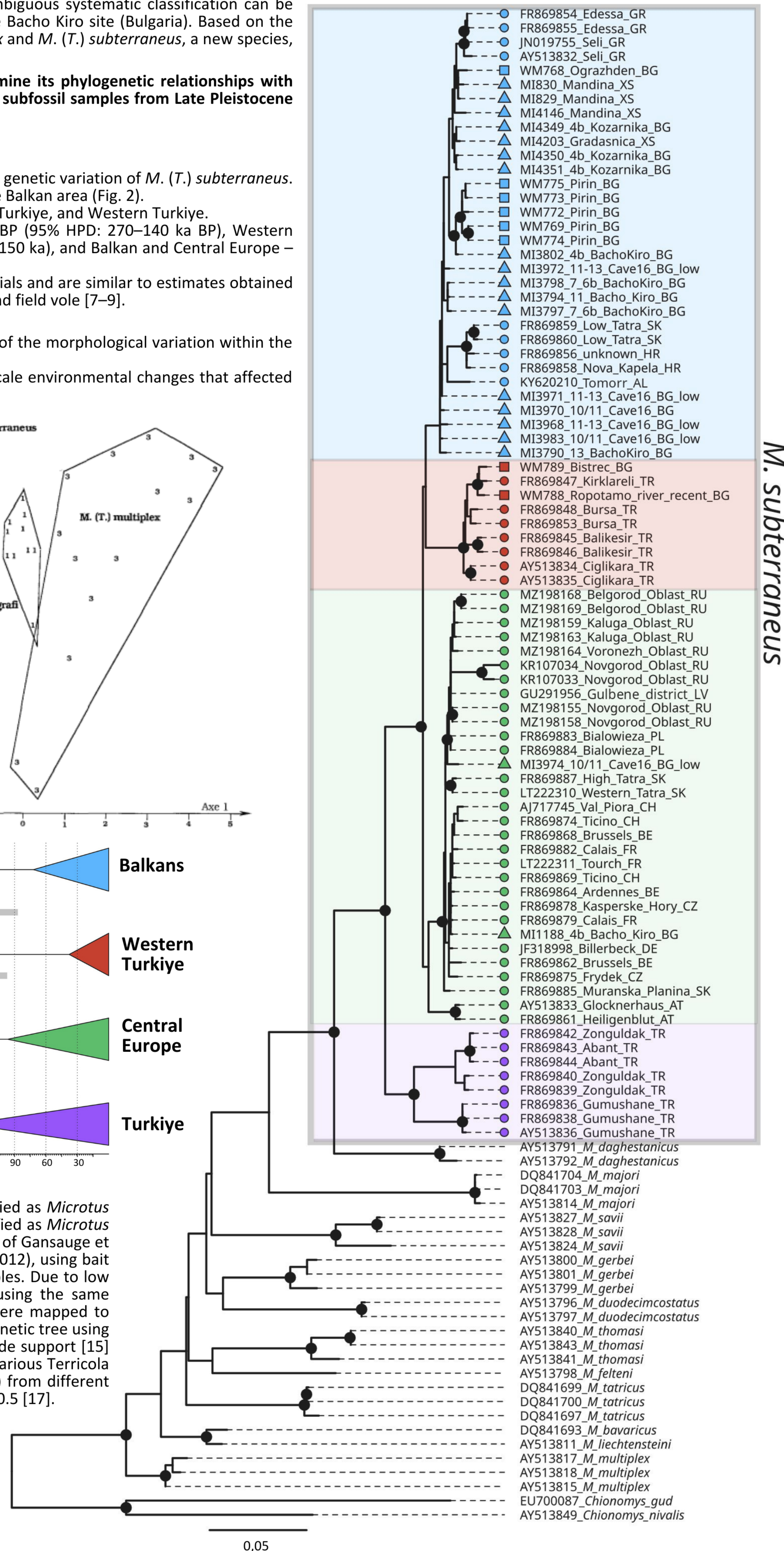


Figure 2. Phylogenetic tree of the subgenus *Terricola*, based on mtDNA cytochrome *b* and rooted using *Chionomys* sp. sequences. Nodes with ultrafast bootstrap and Bayesian posterior probability >90 are marked with circles. Lineages and sample origins are color- and shape-coded as described in Fig. 1b. *M. (T.) subterraneus* samples are indicated by a grey box.